

## The Primary Substrate Binding Site in the b' Domain of ERp57 Is Adapted for Endoplasmic Reticulum Lectin Association\*

Received for publication, January 20, 2004  
Published, JBC Papers in Press, February 10, 2004, DOI 10.1074/jbc.M400575200

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ERp57 is a member of the protein disulfide isomerase (PDI) family that is located in the endoplasmic reticulum (ER) and characterized by its specificity for glycoproteins. Substrate selection by ERp57 is dependent upon its formation of discrete complexes with two ER resident lectins, soluble calreticulin and membrane-bound calnexin. It is these two lectins that directly associate with glycoproteins bearing correctly trimmed oligosaccharide side chains. Thus, ERp57 is presented with a preselected set of substrates upon which it can act, and the specific binding of calreticulin and calnexin to ERp57 is pivotal to the functions of the resulting complexes. To gain further insights into the formation of these ERp57-ER lectin complexes, we have investigated the regions of ERp57 that are specifically required for its binding to calreticulin. Using a quantitative pull-down assay to investigate the binding of ERp57/PDI chimeras to calreticulin, we define the b and b' domains of ERp57 as the minimal elements that are sufficient for complex formation. This analysis further identifies a novel role for the distinctive C-terminal extension of ERp57 in reconstituting complex formation to wild type levels. Using our understanding of substrate binding to the b' domain of PDI as a paradigm, we show that alterations to specific residues in the b' domain of ERp57 dramatically reduce or completely abolish its binding to calreticulin. On the basis of these data, we propose a model where the region of ERp57 equivalent to the primary substrate binding site of archetypal PDI is occupied by calreticulin and suggest that the ER lectins act as adaptor molecules that define the substrate specificity of ERp57.

The ER<sup>1</sup> contains a number of molecular chaperones and folding factors that facilitate the folding and assembly of newly synthesized polypeptides as they are translocated across the ER membrane and into its lumen. Among these, a set of glycoprotein-specific components have been identified and found to be dependent upon the covalent attachment of N-linked oligosaccharides to nascent polypeptides (1, 2). This recruitment process is tightly regulated by the glucose trimming of the N-linked glycans such that the monoglucosylated form of the oligosaccharide binds to two homologous ER lectins, calnexin and calreticulin, that are pivotal to the chaperone-mediated folding of glycoproteins (1, 2). There is evidence that the integral membrane protein calnexin, and the soluble luminal protein calreticulin, can act as *bona fide* molecular chaperones, and hence might influence protein folding directly (3, 4). However, a primary function of calnexin and calreticulin is to bring newly synthesized glycoproteins together with the protein disulfide isomerase (PDI)-like protein ERp57, and these ER lectin-ERp57 complexes significantly promote native disulfide bond formation in their glycoprotein substrates (5, 6). In practice, ERp57 has been shown to form stable complexes with both calnexin and calreticulin, and these complexes can be readily detected using an *in vitro* cross-linking assay (7).

High resolution structures for substantial segments of calreticulin and calnexin are available (8–10). Further studies show that ERp57 binds to the tip of the P-domain of calreticulin (11, 12), an extended hairpin fold that protrudes from the compact globular domain containing the lectin activity and that is conserved in calnexin (10). A second, zinc-dependent interaction of ERp57 with the globular domains of these ER lectins can also be detected *in vitro* (12). In contrast, the one or more regions of ERp57 that enable it to assemble specifically with calnexin and calreticulin are unclear (*cf.* Ref. 7).

All of the members of the PDI family are characterized by a modular structure, and both PDI and ERp57 have the same basic domain organization of a, b, b', a', and c where the a and a' domains contain the thioredoxin-like motif Cys-Xaa-Xaa-Cys (see Ref. 13). For PDI, simple thiol-disulfide chemistry only requires the a or a' domains, and simple isomerization requires one of these active domains in combination with the b' domain (14, 15). However, complex isomerization requires all four PDI

\* This work was supported by the award of a Biotechnology and Biological Sciences Research Council (BBSRC) Professorial Fellowship (to S. H.), a BBSRC studentship (to S. J. R.), a University of Wales Ph.D. studentship (to H. L. R.), funding from the Biocenter Oulu (to L. W. R.), and grants from the Health Sciences Council (200471) and the Finnish Centre of Excellence Programme 2000–2005 (44843) of the Academy of Finland (to J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: ER, endoplasmic reticulum; BMH, bis-maleimidohexane; ERp57 $\Delta$ C, ERp57 lacking its C-terminal region (encodes residues Met<sup>1</sup> to Ile<sup>490</sup>); ERp57 $\Delta$ ss, ERp57 lacking its signal sequence and starting at an initiator Met followed by residue Ser<sup>25</sup> of the full-length coding region; PDI, protein disulfide isomerase; TBS, Tris-buffered saline; CD, circular dichroism; TX-100, Triton X-100.

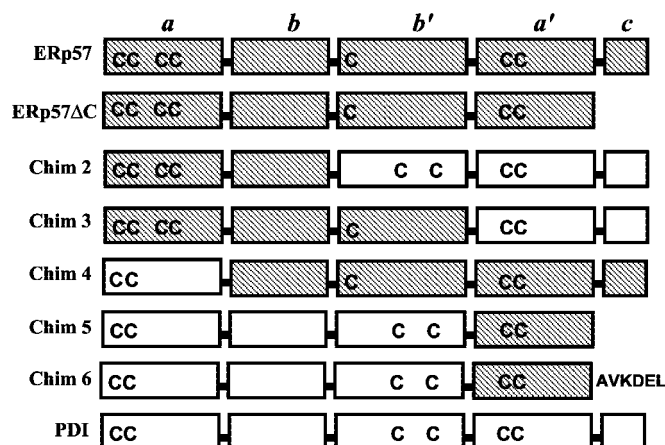


FIG. 1. Outline of the origins of the ERp57/PDI chimeras used in this study. Domains (as defined in Ref. 13) derived from ERp57 are stippled, whereas those from PDI are in white. The number and approximate location of each cysteine residue present in the mature (signal sequence cleaved) polypeptides is indicated. The various derivatives have the ER signal sequence from the N-terminal polypeptide and then the following residues (numbering refers to full-length human ERp57 and PDI sequences, including their respective ER signal sequences): ERp57 = Ser<sup>25</sup>-Leu<sup>505</sup>, ERp57ΔC = Ser<sup>25</sup>-Ile<sup>490</sup>, chimera 2 = ERp57 Ser<sup>25</sup>-Gly<sup>242</sup> and PDI Leu<sup>236</sup>-Leu<sup>508</sup>, chimera 3 = ERp57 Ser<sup>25</sup>-Gly<sup>376</sup> and PDI Pro<sup>368</sup>-Leu<sup>508</sup>, chimera 4 = PDI Asp<sup>18</sup>-Gly<sup>134</sup> and ERp57 Pro<sup>134</sup>-Leu<sup>505</sup>, chimera 5 = PDI Asp<sup>18</sup>-Gln<sup>367</sup> and ERp57 Pro<sup>377</sup>-Val<sup>489</sup>, chimera 6 = same as chimera 5 plus the sequence AVKDEL (*cf.* Ref. 19). Note that mature ERp57 starts at Ser<sup>25</sup> and PDI at Asp<sup>18</sup>.

domains (abb'a'), but not the C-terminal region (14, 15). The b' domain was found to be essential for PDI-protein substrate interactions and is sufficient for the binding of small peptides, although other domains contributed to the association of PDI with large peptides and partially unfolded proteins (16–18). Hence, the b' domain of PDI appears to be its principle peptide binding site, although other domains may be required for interactions with larger substrates. The conserved modular structure of PDI and ERp57 has been exploited to generate chimeras that have then been used to define the one or more regions of PDI that facilitate specific interactions with other ER proteins to generate protein complexes with defined functions. Using this approach it was found that both the b' and a' domains of PDI are the minimum requirement for it to function as a subunit of collagen prolyl 4-hydroxylase and that the inclusion of the b domain enhances this activity significantly (19).

We have previously shown that ERp57 associates specifically with the two ER lectins, calnexin and calreticulin, whereas archetypal PDI does not (7). To establish the molecular basis for the specific assembly of ERp57 with calnexin and calreticulin, we have exploited chimeras derived from the two related proteins to map the domains of ERp57 that mediate its binding to the ER lectins. Using a newly established pull-down assay, we find that the principal binding site for calreticulin lies within the region defined by the b and b' domains of ERp57 and show that its distinct C-terminal region enhances ERp57 binding. To more closely define the binding of calreticulin to ERp57, we have exploited our knowledge of substrate binding to PDI. Using previously characterized point mutants of the PDI b' domain as a starting point (18), we show that the alteration of equivalent residues in the ERp57 b' domain can completely abolish its binding to calreticulin while still allowing the protein to attain a structure comparable to that of the wild type protein. On the basis of these data we propose that regions of ERp57 equivalent to the substrate binding site of PDI are occupied by calnexin or calreticulin, and these lectins act as

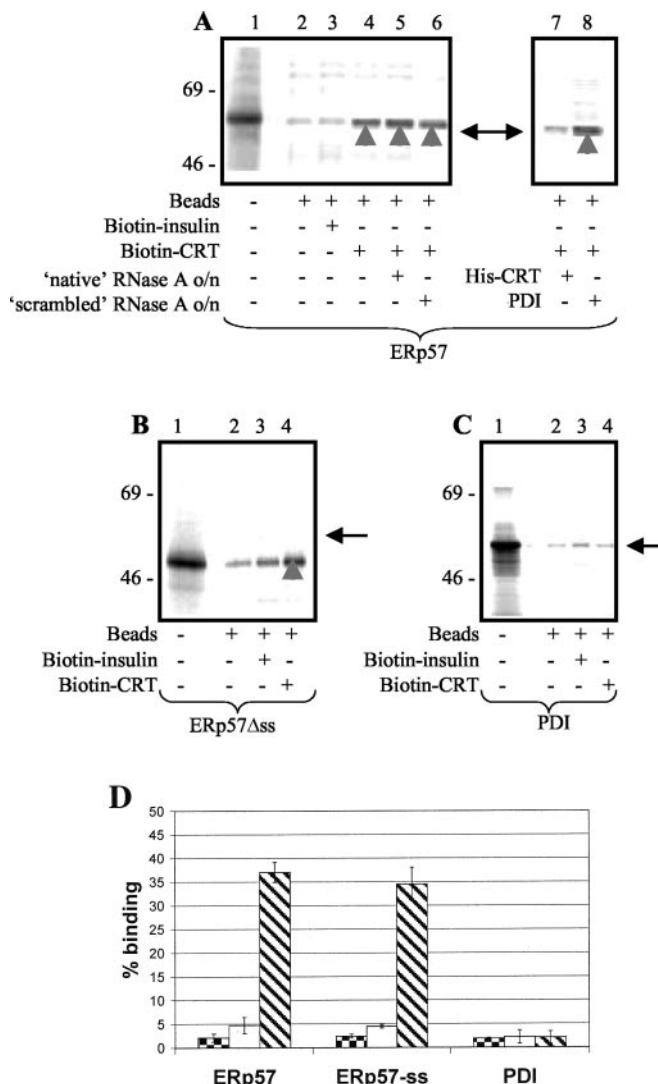
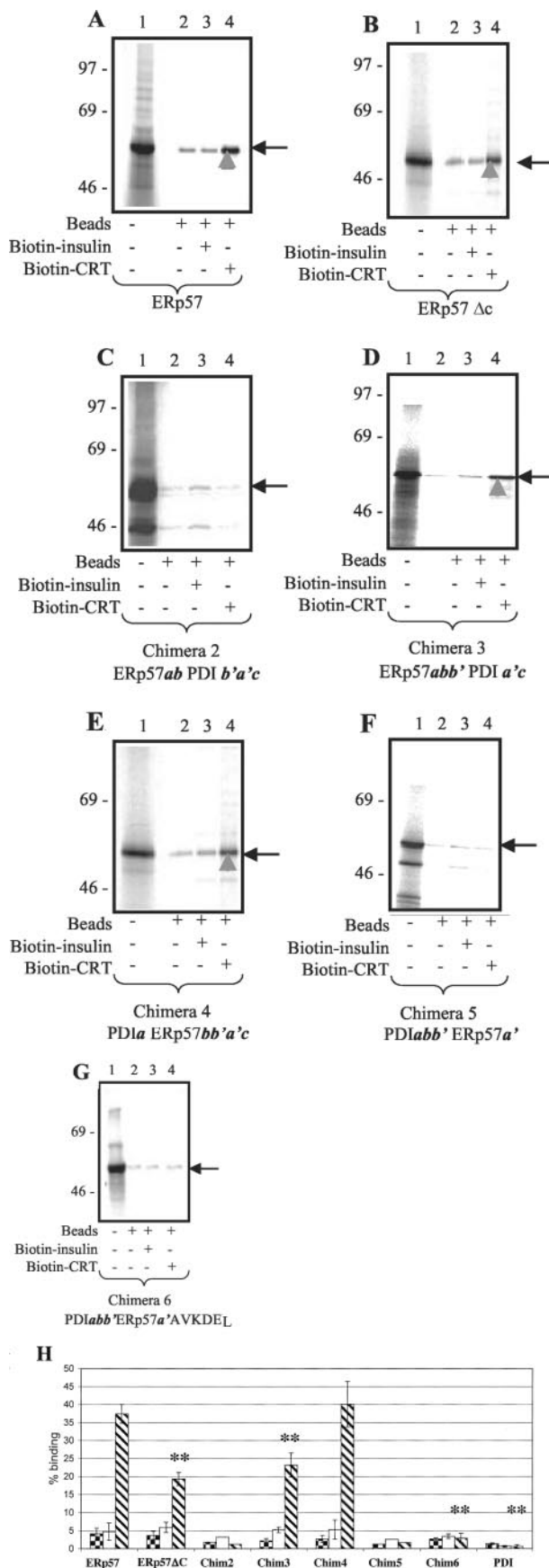


FIG. 2. ERp57 binds specifically to immobilized calreticulin. ERp57 (A), ERp57Δss (B), and PDI (C) were synthesized as <sup>35</sup>S-labeled polypeptides in reticulocyte lysate and then mixed with NeutrAvidin beads alone (A–C, lane 2), NeutrAvidin beads loaded with biotin-tagged insulin (A–C, lane 3), or NeutrAvidin beads loaded with biotin-tagged calreticulin (A–C, lane 4). In the case of ERp57, additional incubations using NeutrAvidin beads loaded with biotin-tagged calreticulin and preincubated with either “native” or “scrambled” RNase A (A, lanes 5 and 6, respectively) were included, as were samples where His-tagged calreticulin or recombinant PDI were added in conjunction with radiolabeled ERp57 (A, lanes 7 and 8, respectively). The percentage of the total radiolabeled input that was recovered bound to the beads was calculated by quantitative phosphorimaging using an equal fraction of the translation products for comparison (A–C, lane 1). Levels of radiolabeled material that are significantly above background are indicated by a gray arrowhead (*cf.* D). D, the basic binding assay was repeated twice and the average value and standard error for the proportion of material recovered with beads alone (checkerboard bar), beads loaded with biotinylated insulin (white bar), and beads loaded with biotinylated calreticulin (hatched bar) are shown.

adaptors that specifically recruit glycoprotein substrates to the ERp57-ER lectin chaperone complexes.

#### EXPERIMENTAL PROCEDURES

**Materials**—Bismaleimidoethane (BMH) was purchased from Pierce and Warriner (Chester, UK), restriction endonucleases were from New England Biolabs (Herts, UK), and RNA polymerase, transcription reagents, and rabbit reticulocyte lysate were from Promega (Southampton, UK). Easytag L-[<sup>35</sup>S]methionine was obtained from PerkinElmer Life Sciences (Stevenage, UK), whereas all other chemicals were from BDH/Merck (Poole, UK) and Sigma (Poole, UK). The rabbit antiserum specific for the C-terminal region of human ERp57



**FIG. 3. Binding of ERp57 derivatives to immobilized calreticulin.** ERp57 (A), ERp57ΔC (B), and chimeras 2–6 (C–G) were synthesized as  $^{35}\text{S}$ -labeled polypeptides in reticulocyte lysate and analyzed for binding to immobilized calreticulin as described in the legend to Fig. 2. Levels of radiolabeled material that are significantly above background

was generated to order by Eurogentec (Seraing, Belgium), whereas the anti-calreticulin serum was obtained from Cambridge Bioscience (Cambridge, UK). Purified, recombinant PDI was a gift from Professor N. Bulleid, University of Manchester, UK.

**ERp57 and PDI Derivatives**—Wild type human ERp57 and human PDI were as previously described (7). PDI/ERp57 chimeric constructs 2–6 (cf. Ref. 19) were removed from the pVL1392 vector by restriction digestion and then ligated into either pBluescript KS II or pBluescript SK II under the control of the T7 promoter (cf. Ref. 7). The ERp57Δss construct was generated by using PCR to remove the region encoding the signal sequence and to introduce an ATG start codon immediately before that encoding the first residue of the mature sequence. The precise origin of each ERp57/PDI chimera is indicated in the legend to Fig. 1. The thirteen ERp57 b' domain point mutants (V267A, V267W, Y269A, Y269W, E270A, K274A, R280A, N281A, V283A, V283L, V283W, F299A, and F299W) were generated by mutagenesis of the wild type coding region present in pBluescript KS II, and all mutants were authenticated by DNA sequencing.

**Cross-linking Analysis**—The ability of wild type ERp57, and the various b' domain point mutants, to associate with the endogenous calreticulin present in ER-derived canine pancreatic microsomes was carried out essentially as previously described (7). A flexi rabbit reticulocyte lysate system was used to synthesize the polypeptides at 30 °C for 1 h, and translation was terminated by treatment with 2 mM puromycin. Cross-linking with BMH (final concentration, 500 μM) and product identification by immunoprecipitation were also as previously detailed (7).

**Preparation of Biotinylated Calreticulin**—A calreticulin cDNA fragment lacking the ER signal sequence was amplified by PCR from a plasmid containing the full-length cDNA (20) utilizing a modified sense primer incorporating an NruI site at its 5' end to facilitate its cloning downstream of the biotin purification tag present in the Pin-Point Xa-1 vector (Promega). Sequencing of the recombinant Pin Point construct confirmed that the mature calreticulin coding region was inserted adjacent to, and in frame with, the plasmid fusion tag with an intact factor Xa cleavage site. *E. coli* strain JM109 harboring the expression plasmid was grown to an optical density of 0.4–0.6 in media supplemented with 2 mM biotin, and expression of the fusion protein was induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Cells were harvested, lysed in the presence of a protease inhibitor mixture (Sigma), and the resulting cell lysate passed over a SoftLink avidin resin as described by the manufacturers (Promega). Biotin-tagged recombinant protein was eluted with buffer containing a gradient of 5 to 20 mM Biotin and exchanged into 20 mM HEPES, pH 7.5, 1 mM EDTA, 50 mM NaCl by dialysis. Samples were tested by Western blotting using streptavidin-coupled horseradish peroxidase, and fractions containing the majority of the biotin-tagged calreticulin were pooled. The final concentration of biotin-tagged calreticulin was determined using the DC Bio-Rad Protein Assay (Bio-Rad, Hemel Hempstead, UK).

**Expression and Purification of His-tagged Proteins**—A version of calreticulin lacking its ER signal sequence, and with an N-terminal 10× His tag, was generated in the vector pET16b and incorporated a factor Xa cleavage site between the poly-His tag and the calreticulin polypeptide. The recombinant protein was expressed in the *Escherichia coli* origami (DE3) strain (Novagen, Nottingham, UK) and purified under native conditions as recommended by the manufacturer (Invitrogen, Paisley, UK). Mature, wild type ERp57 was cloned into a modified version of pET23b, which encodes for the sequence MHHHHHHM, prior to the first amino acid of the mature sequence (21), and selected point mutants generated in this construct were expressed in *E. coli* strain BL21(DE3)pLysS grown either in LB media or in M9 minimal media at 37 °C, 200 rpm and induced at an  $A_{600}$  of 0.3 for 3 h with 1 mM isopropyl β-D-thiogalactoside. Cell lysis and protein purification were carried out as per the purification of ERp18 (22). The concentration of each protein was determined spectrophotometrically using a calculated

are indicated by a gray arrowhead (cf. H). In some cases, the assay was carried out two (chimera 6) or four times (PDI, ERp57, ERp57ΔC, and chimeras 3 and 4), and a summary of these data are presented in panel H. Where appropriate, the average value and standard error for the proportion of material recovered with beads alone (checkerboard bar), beads loaded with biotinylated insulin (white bar), and beads loaded with biotinylated calreticulin (hatched bar) are shown. Calreticulin binding of an ERp57 derivative that is different from that of the wild type protein to a significance of 0.01 is shown by a double asterisk. There is no significant difference between the values obtained for ERp57ΔC and chimera 3.



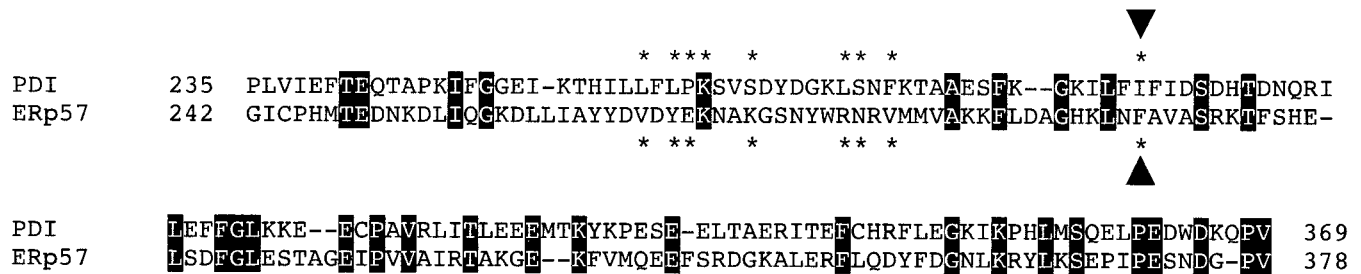


FIG. 4. Alignment of the region between the b and a' domains of human PDI and ERp57. Identical residues are highlighted in black, numbering is for the full-length proteins. All gaps in the PDI alignment lie between secondary structural elements in the published model of the b' domain (18). Asterisks above the alignment indicate sites of mutations reported to effect PDI substrate binding (18); asterisks below the alignment indicate sites of mutations made in this study in ERp57. The locations of Ile<sup>289</sup> in PDI and Phe<sup>299</sup> in ERp57 are also indicated by arrowheads.

absorption coefficient of 45,200 M<sup>-1</sup> cm<sup>-1</sup> (molecular weight of 55,276) for the wild type protein.

**ERp57 Pull-down Assay**—UltraLink NeutrAvidin beads (Pierce) were preincubated with 20% bovine serum albumin to block any non-specific binding sites and washed 5 times in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). In some cases, biotin-tagged calreticulin (5 µg), or an equimolar amount of biotin-tagged insulin (~0.6 µg), was then immobilized on these beads by mixing overnight at 4 °C in the presence of 100 µM phenylmethylsulfonyl fluoride and 0.25% TX-100 in TBS. Control reactions also included a 10-fold molar excess of "native" or "scrambled" RNase A (~1.4 µg), during the overnight incubation. The beads were recovered by centrifugation for 2 min at 3,000 rpm in a microcentrifuge and washed three times in 0.25% TX-100 in TBS. 10 µl of a specific *in vitro* translation reaction, carried out in the absence of canine pancreatic microsomes and pre-spun at 1000 × *g* for 2 min, was then mixed with 20 µl of NeutrAvidin beads alone, 20 µl of NeutrAvidin beads loaded with biotin-tagged insulin, or 20 µl of NeutrAvidin beads loaded with biotin-tagged calreticulin. Competition experiments were performed together with a 10-fold molar excess of His-tagged calreticulin (25 µg) or recombinant PDI (~30 µg). 50 µl of TBS with 0.25% (v/v) TX-100 was added to each sample, they were mixed at room temperature for 2 h, and the beads were then recovered by centrifugation, washed four times in TBS with 0.25% (v/v) TX-100, and then once with TBS.

**SDS-PAGE Analysis**—Samples were denatured and solubilized in SDS sample buffer by heating to 70 °C for 10 min before being resolved on SDS-PAGE gels of 8% (cross-linking products) or 15% (pull-down assays) acrylamide. Gels were fixed, exposed to a phosphorimaging plate, and visualized on a Fuji BAS-1800 bioimaging system. Quantification was carried out using AIDA software supplied by Raytek Scientific Ltd. (Sheffield, UK). Where shown, statistical significance was determined by using a two-tailed Student's *t* test (Microsoft Excel).

**Biophysical Analysis**—Far-UV circular dichroism spectra were recorded on a Jasco J600 spectrophotometer. All spectra were collected at 25 °C as an average of 8 scans, using a cell with a path length of 0.1 cm, scan speed of 20 nm/min, a spectral bandwidth of 1.0 nm, and a time constant of 1 s. The maximal high tension voltage was 750 V. All spectra were corrected for the blank spectra with no protein added.

## RESULTS

Although ERp57 and PDI share significant sequence identity and most likely have similar tertiary structures (13), we have previously shown that ERp57 forms stable complexes with calnexin and calreticulin, whereas PDI does not (7). In this study we have exploited a series of chimeras of PDI and ERp57 (19), together with other ERp57 derivatives and point mutants, to define the domains of ERp57 that are required for its specific association with the ER lectins calreticulin and calnexin.

**Only a Subset of ERp57 Derivatives Bind to Calreticulin in an *in Vitro* Pull-down Assay**—Our previous studies of ERp57 binding to ER lectins relied on a cross-linking assay (7) that may be influenced by differences in the number and location of the cysteine residues present in the various chimeras (see Fig. 1). Furthermore, such assays are unable to formally distinguish binding from close proximity. We therefore established a quantitative "pull-down" assay that reflected authentic binding between two components to investigate the association between ERp57 and the ER lectin calreticulin. Recombinant cal-

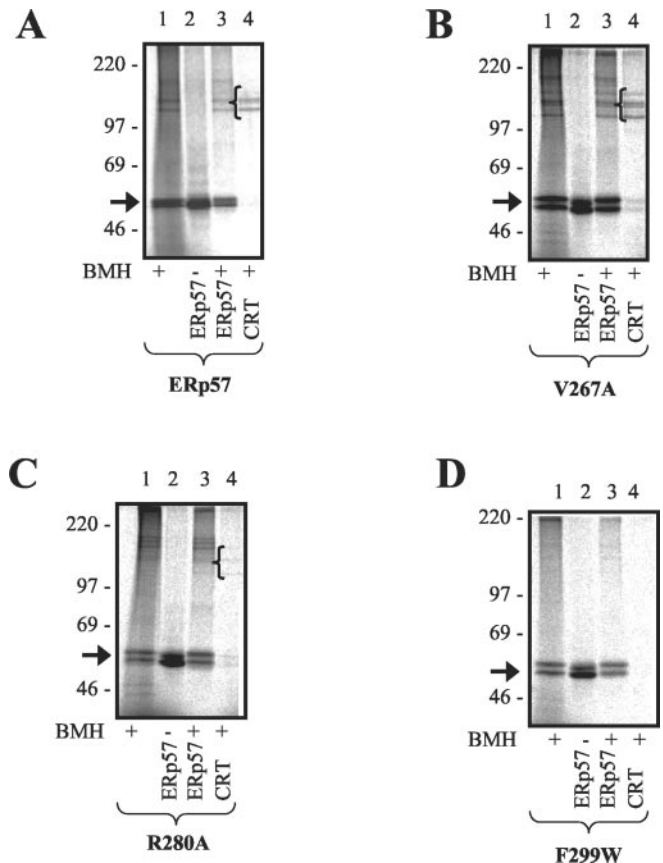


FIG. 5. Cross-linking of wild type ERp57 and b' domain point mutants to the endogenous calreticulin present in canine pancreatic microsomes. mRNAs encoding wild type (A) or various point mutants of ERp57 (B–D) were translated in a rabbit reticulocyte lysate translation system in the presence of canine pancreatic microsomes, treated with BMH, and analyzed by immunoprecipitation. In this case total products after cross-linking, products immunoprecipitated by the anti-ERp57 serum with and without cross-linking, and products immunoprecipitated with anti-calreticulin after cross-linking were analyzed. ERp57 polypeptides are indicated by a black arrow, whereas adducts with calreticulin are denoted by a brace. The efficiency of cross-linking for each ERp57 derivative to calreticulin was estimated using phosphorimaging. The total amount of radiolabeled material present in the anti-calreticulin immunoprecipitation (lane 4) was quantified and expressed as a percentage of the total radiolabeled ERp57 recovered in the anti-ERp57 immunoprecipitation without BMH treatment (lane 2). Representative examples of different ERp57 b' mutants are shown, and the quantification results for all of the mutants analyzed are shown in Table I.

reticulin, containing an N-terminal biotinylated tag, was immobilized on tetrameric NeutrAvidin-coated beads and used as a ligand for <sup>35</sup>S-labeled ERp57 and its derivatives that were generated by *in vitro* translation. When wild type ERp57 was

incubated with immobilized calreticulin, a little over one-third of the radiolabeled protein was specifically recovered with the beads (Fig. 2A, cf. lanes 1 and 4, Fig. 2D). In contrast, only background levels of binding (~3–5%) were found when beads alone or beads loaded with biotinylated insulin were used (Fig. 2A, cf. lanes 1–3, see also Fig. 2D).

Preincubation of the calreticulin bait with a 10-fold molar excess of scrambled or native RNase A caused either a minor reduction in binding (to ~30%; cf. 37%) or had no significant effect (~38%; cf. 37%), respectively (Fig. 2A, cf. lanes 4–6, Fig. 2D). Thus, ERp57 binding to immobilized calreticulin bears the hallmark of complex formation rather than the binding of a potentially misfolded protein (ERp57) to a candidate molecular chaperone (calreticulin). As expected, a 10-fold molar excess of soluble recombinant His-tagged calreticulin efficiently competed with the immobilized biotinylated calreticulin for ERp57 binding, whereas a similar molar excess of recombinant His-tagged PDI did not (Fig. 2A, lane 7, ~5%; lane 8, ~30%). Our previous cross-linking analysis indicated that the presence of an uncleaved signal sequence at the N terminus of ERp57 did not affect its ability to bind to calreticulin (7). We confirmed that this was the case by analyzing an ERp57 derivative lacking its N-terminal signal sequence (ERp57Δss) in the pull-down assay and finding that ERp57-calreticulin complex formation was essentially unaffected (Fig. 2, cf. A, B, and D). The specificity of the interaction between radiolabeled ERp57 and immobilized, biotinylated calreticulin was further underscored by the observation that only ~2% of radiolabeled PDI was recovered in this pull-down assay under any of the various conditions tested (Fig. 2, C and D).

Having established a robust pull-down assay, various ERp57 chimeras were investigated. Radiolabeled versions of ERp57ΔC and five chimeras (see Fig. 1, constructs 2–6) were synthesized *in vitro* and analyzed using the pull-down assay outlined above. As before, a substantial fraction of ERp57 (37%) was bound to beads loaded with biotin-tagged calreticulin but not to control beads (cf. Fig. 3A, lanes 2–4, Fig. 3H). When the ERp57ΔC polypeptide and the various ERp57/PDI chimeras were tested using the pull-down assay, it was immediately apparent that only a subset of these proteins displayed significant binding to the immobilized calreticulin (Figs. 3, B–H). Hence, although ERp57ΔC and chimeras 3 and 4 showed levels of specific binding substantially above the levels observed in matched controls (Fig. 3, B, D, E, and H), no such binding was detected with constructs 2, 5, or 6 (Fig. 3, C, F, G, and H).

Quantification of the pull-down data (see Fig. 3H) showed that, although wild type ERp57, ERp57ΔC, and chimeras 3 and 4 all showed significant levels of binding to calreticulin, there was a clear and statistically significant difference in the efficiency of binding between the various constructs. Thus, only chimera 4 showed binding equivalent to wild type ERp57 (~40 and ~37% respectively, see Fig. 3H), whereas the binding of both ERp57ΔC and chimera 3 were both significantly reduced (~19 and ~23% respectively, see Fig. 3H). Thus, the b and b' domains of ERp57 are the minimal elements necessary for calreticulin binding that are defined by this analysis (cf. Fig. 1). Furthermore, although the presence of the C-terminal region is not absolutely required for complex formation, it is required for levels of binding akin to that seen with the wild type protein.

**Generation of ERp57 b' Domain Point Mutants and Their Analysis by Cross-linking**—The b' domain of PDI has been shown to play a major role in the binding of peptide substrates (16–18), and the b' domains of ERp57 and PDI show substantial sequence divergence from one another (24% sequence identity, see Fig. 4; cf. 49% for the a domains, see Ref. 23). Our analysis of the ERp57/PDI chimeras indicated that the princi-

TABLE I  
Percentage of ERp57 b' domain point mutants found cross-linked to endogenous calreticulin

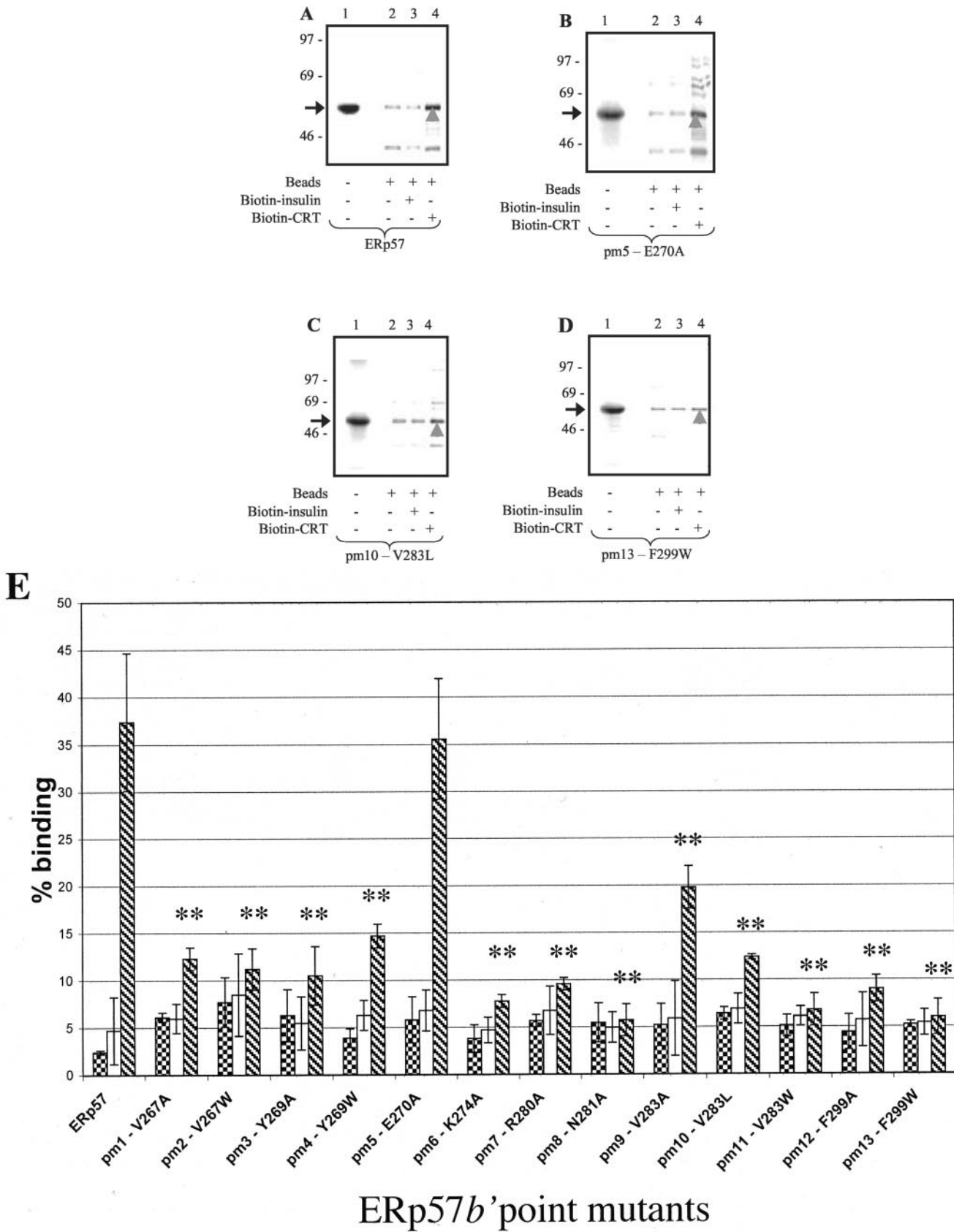
ERp57 b' domain mutant	WT <sup>a</sup>
	%
Wild type	100
Val <sup>267</sup> → Ala	83
Val <sup>267</sup> → Trp	83
Tyr <sup>269</sup> → Ala	100
Tyr <sup>269</sup> → Trp	83
Glu <sup>270</sup> → Ala	100
Lys <sup>274</sup> → Ala	83
Arg <sup>280</sup> → Ala	33
Asn <sup>281</sup> → Ala	83
Val <sup>283</sup> → Ala	67
Val <sup>283</sup> → Leu	33
Val <sup>283</sup> → Trp	67
Phe <sup>299</sup> → Ala	33
Phe <sup>299</sup> → Trp	<17

<sup>a</sup> The fraction of the membrane-associated ERp57 derivative that was cross-linked to the endogenous calreticulin present in canine pancreatic microsomes was established by quantitative phosphorimaging (see also Fig. 5). These values were then expressed as a percentage of that found with the wild type protein (%WT).

pal calreticulin binding site was located somewhere in its b and b' domains, and we hypothesized that the b' domain of ERp57 might make a major contribution to the calreticulin binding site analogous to the role of the PDI b' domain in substrate binding. To investigate this possibility further, a number of targeted point mutations based on residues important for substrate binding to PDI (18) were introduced into the b' domain of ERp57. The mutations were selected based on the alignment of the b' domains from ERp57 and PDI (see Fig. 4) and the recently reported identification of the primary substrate binding site within the b' domain of human PDI (18). In particular, several mutations were made in the four residues (Val<sup>267</sup>, Tyr<sup>269</sup>, Val<sup>283</sup>, and Phe<sup>299</sup>) analogous to those found to define the binding site in PDI, whereas single mutations to alanine were made in residues analogous to those juxtaposed to this site (Glu<sup>270</sup>, Lys<sup>274</sup>, Arg<sup>280</sup>, and Asn<sup>281</sup>).

The ability of the various ERp57 b' domain mutants to associate with calreticulin was initially screened using a previously established cross-linking assay (7). In all cases the ERp57 point mutants were efficiently synthesized and imported into microsomes (Fig. 5, A–D, data not shown). For most of the point mutants, some BMH-dependent cross-linking products were visible, although the intensity of these adducts was variable (e.g. Fig. 5, A–D, data not shown, see also Table I). Immunoprecipitation confirmed that most, although not all, of the point mutants formed distinct adducts with calreticulin (Fig. 5, A–D, lane 4; and data not shown). Where visible, multiple ERp57/calreticulin adducts were observed as previously described for the wild type protein (7). These multiple products most likely reflect cross-linking between different cysteine residues present within ERp57 and calreticulin (7). To compare the efficiency of cross-linking for the different b' point mutants, the relative proportion of ERp57 cross-linked to calreticulin was determined for each of the mutants and expressed as a percentage of the value obtained with the wild type protein (see Table I). This quantitative analysis suggested that there was a significant variation in the efficiency of cross-linking of the ERp57 point mutants to calreticulin, with the F299W mutant generating no clearly discernable adducts (see Fig. 5D, lane 4, and Table I). We conclude that specific residues in the b' domain of ERp57 (Arg<sup>280</sup>, Val<sup>283</sup>, and Phe<sup>299</sup>) are important for its ability to be cross-linked to calreticulin efficiently.

**Pull-down Analysis of ERp57 b' Domain Point Mutants**—Because the majority of the ERp57 b' domain point mutants



**FIG. 6. The binding of ERp57 b' domain point mutants to immobilized calreticulin.** ERp57 (A) and point mutants 5-Glu<sup>270</sup> → Ala (B) 10-Val<sup>283</sup> → Leu (C), and 13-Phe<sup>299</sup> → Trp (D) were synthesized as <sup>35</sup>S-labeled polypeptides in reticulocyte lysate and analyzed for binding to immobilized calreticulin as described in the legend to Fig. 2. The radiolabeled ERp57 that was recovered with the calreticulin-loaded beads is indicated by a gray arrowhead. All of the point mutants listed in Table I were analyzed in this way, and a representative set of mutants is presented. For each ERp57 b' mutant the assay was repeated three times, and the average value and standard error for the proportion of material recovered with beads alone (checkerboard bar), beads loaded with biotinylated insulin (white bar), and beads loaded with biotinylated calreticulin (hatched bar) are shown in panel E. Calreticulin binding of an ERp57 b' domain mutant that is different from that of the wild type protein to a significance of 0.01 is shown by a double asterisk.



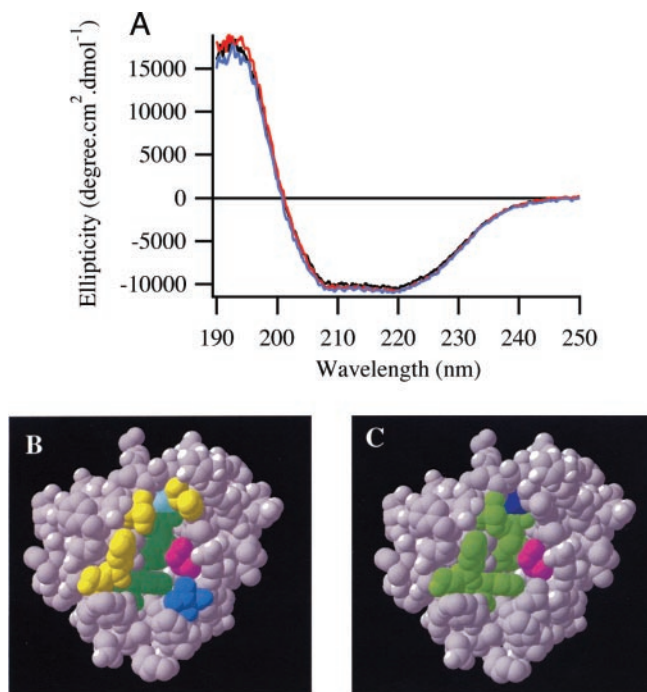
displayed an ability to be cross-linked to the endogenous calreticulin present in ER-derived microsomes (Table I), the thirteen point mutants were all analyzed for calreticulin binding using the pull-down assay (Fig. 6, A–E, data not shown). Significantly, the analysis of the b' mutants using the pull-down assay revealed that almost all of the point mutants had a severe defect in calreticulin binding (Fig. 6E). Quantification showed that only one of the thirteen point mutants analyzed, ERp57-Glu<sup>270</sup> → Ala, bound to immobilized calreticulin with an efficiency similar to the wild type protein (~35 and ~37%, respectively, Fig. 6, A, B, and E). A second mutant, Val<sup>283</sup> → Ala, exhibited an intermediate level of binding (~20%, Fig. 6E), whereas three other mutants, Val<sup>267</sup> → Ala, Tyr<sup>269</sup> → Trp, and Val<sup>283</sup> → Leu (see Fig. 6C), all showed levels of binding that were significantly above their matched controls but substantially reduced in comparison to the wild type protein (~12, ~15, and ~12%, respectively, see Fig. 6E). All of the other point mutants displayed levels of binding that were not significantly above the background level (Fig. 6, D and E). Taken together, the pull-down analysis of the b' domain point mutants is consistent with a model where this region of ERp57 plays a pivotal role in its stable association with the ER lectin calreticulin.

The mutations that were introduced into the ERp57 b' domain were based upon previous studies of PDI where comparable alterations had been shown to cause minimal perturbation to the structure of the protein. To investigate this issue further for ERp57, we purified two of the mutants with a strong defect in calreticulin binding, the Phe<sup>299</sup> → Ala and Phe<sup>299</sup> → Trp mutants, and analyzed them using CD spectroscopy. Both mutants showed near-identical CD spectra with the wild type protein (Fig. 7A), indicating that there were no gross changes in their respective structures.

#### DISCUSSION

During the course of this study we have investigated the molecular basis for the specific interaction between the PDI-like protein ERp57 and the soluble ER lectin calreticulin. ERp57/PDI chimeras and ERp57 b' domain point mutants were synthesized *in vitro*, and their binding to endogenous ER lectins and recombinant calreticulin was determined. Chimeras 2–6 (cf. Fig. 1) have previously been used to study collagen prolyl 4-hydroxylase complex formation, where PDI functions as the  $\beta$  subunit (19). This study indicated that the chimeras could attain a native-like conformation and, in the cases of ERp57aPDIbb'a'c and ERp57abPDIb'a'c, form active collagen prolyl 4-hydroxylase complexes (19).

**Pull-down Analysis Maps the Calreticulin Binding Site to within the b and b' Domains of ERp57**—Previous studies indicated that ERp57 could bind to calreticulin in solution (7, 24) and showed this interaction was crucial for ERp57 function (5). The estimated dissociation constant for the complex ( $9.1 \pm 3.0 \times 10^{-6}$  M) (11) suggested that binding may be strong enough to survive a pull-down assay, and we found that this was the case. Hence, a substantial proportion of wild type ERp57 was specifically recovered bound to immobilized calreticulin, whereas no specific binding of PDI was observed in the same assay. Likewise, the binding of radiolabeled ERp57 to the immobilized calreticulin bait could be prevented by an excess of soluble calreticulin but not of PDI. Previous studies indicate that calnexin and calreticulin can bind non-glycosylated proteins both *in vitro* and *in vivo* (3, 4, 25), perhaps reflecting a role as chaperones that is additional to their lectin function. However, when either scrambled or native RNase A was incorporated into our pull-down assay, neither of these polypeptides competed efficiently with ERp57 for binding to immobilized calreticulin (cf. Ref. 16). We conclude that the binding of ERp57 to immobilized calreticulin reflects the formation of a biologi-



**FIG. 7. Further analysis of ERp57 b' domain mutants.** A, far-UV CD spectra of wild type ERp57 (black line), Phe<sup>299</sup> → Ala (red line), and Phe<sup>299</sup> → Trp (blue line) mutants of ERp57. B, space-filling models illustrating the substrate binding site in human PDI, numbering is for the full-length protein (modified from Ref. 18). Residues whose mutation caused a major decrease in peptide binding are highlighted in green with residue Ile<sup>289</sup> specifically picked out in magenta, residues whose mutation have a minor effect on substrate binding are in yellow, the residue whose mutation had no effect on substrate binding is in blue. Proline 262 whose mutation resulted in the production of no full-length PDI is in cyan (18). C, residues of human PDI that, when the analogous residues are mutated in ERp57 (cf. Fig. 4), have an effect on calreticulin binding. The coloration is as described for panel B.

cally relevant complex and not the binding of a population of misfolded ERp57 polypeptides to calreticulin acting as a broad specificity chaperone.

When ERp57 derivatives were tested in the pull-down assay, only ERp57 $\Delta$ C missing the C-terminal extension (c) and chimeric constructs ERp57abb'PDIa'c and PDIaERp57bb'a'c bound calreticulin at significant levels. The quantitative nature of the assay revealed clear differences in binding, and only PDIaERp57bb'a'c displayed binding equivalent to wild type ERp57. Hence, the a' domain of ERp57 appears to be entirely dispensable for its binding to calreticulin (cf. Fig. 1). This conclusion is consistent with previous studies of the assembly of archetypal PDI into a functional collagen prolyl 4-hydroxylase complex, where the loss or replacement of its a' domain had the least impact upon the activity of the complex (19). The deletion of the C-terminal region alone (ERp57 $\Delta$ C) has the same effect as the substitution of the C-terminal region and the a' domain together (chimera 3), with both derivatives binding roughly half as well as the wild type protein (cf. Fig. 3H). We conclude that it is the lack of the C-terminal region in these two derivatives that causes the reduction in binding we observe and that the a' domain of ERp57 plays no direct role in calreticulin binding. A specialized role for the C-terminal region of ERp57 in calreticulin binding is consistent with its distinct nature (basic) as compared with the more acidic C-terminal region of PDI (26).

**Most ERp57 b' Domain Point Mutants Are Severely Compromised in Calreticulin Binding**—A striking result of our pull-down analysis was that only two of the 13 mutants displayed calreticulin binding levels anywhere near that of the wild type

A

Human	242	I PDPDAKKPEDWIEEMDGEWEPPVI QN
Mouse	242	I PDPDAKKPEDWIEEMDGEWEPPVI QN
<i>C. elegans</i>	237	I PDPDAKKPEDWIDEMDGEWEPPMI DN
<i>Drosophila</i>	242	I PDPDATKPEDWIDEMDGEWEPPMI DN

B

Human	242	GICPHMTEDN-KDLICGKDLLIAYVDVYEKNKCSNYWRNRMMVAKKFLDAGHKLNFAVASRKTFSHE
Mouse	242	GLCPHMTEDN-KDLICGKDLLTAYVDVYEKNKCSNYWRNRMMVAKKFLDAGHKLNFAVASRKTFSHE
<i>C. elegans</i>	237	GFAGIRTQGNLFQFEC-KPIVIVYINVDYVKDPKCSNYWRNRNLKVAQNY--KRVQFAVSNKEEFSSE
<i>Drosophila</i>	234	CLVGHRTQDSVKDF-C-NPLITAYYSVDYKNPKCTNYWRNRNLKVAKEFV--G-QINFATASKDDFQHE
		* * : * * * *

HUMAN	LSDFGL-ESTAGEIPVVAIRTAKEGKFVMQEEFSRDGKALERFLQDYFDGNLKRILKSEPIPESNDGPV	378
MOUSE	LSDFGL-ESTTGEVPVVAIRTAKEGKFVMQEEFSRDGKALEQFLQDYFDGNLKRILKSEPIPESNEGPV	378
<i>C. elegans</i>	LETNGLGERKDSKPTVAILTNEG-KYPMDQEEFSVDN--LQQFVDEVLAGNAEPYMKSEPIPDE-QGDV	364
<i>Drosophila</i>	LNEYCY-D-FVGDKPVVLARDEKNLKYALKDEFSVEN--LQDFVEKLLANELEPIYIKSEPIPESNDAPV	366

FIG. 8. Conserved elements in calreticulin and Erp57. A, an alignment of the region of the P domain of calreticulin that interacts with Erp57 (11) from human, mouse, *C. elegans*, and *Drosophila*. Identical residues are highlighted in black; numbering is for the full-length protein, including the signal sequence. B, alignment of the b' domains of Erp57 from human, mouse, *C. elegans*, and *Drosophila* (based on boundaries defined in Ref. 21). Identical residues are highlighted in black; numbering is for the full-length protein. Residues whose mutation effects human Erp57-calreticulin interactions are indicated by an asterisk below the sequence, Glu<sup>270</sup> whose mutation does not affect these interactions is indicated by the symbol ":" below the sequence.

protein, namely Glu<sup>270</sup> → Ala and Val<sup>283</sup> → Ala. CD spectroscopy showed that mutants Phe<sup>299</sup> → Ala and Phe<sup>299</sup> → Trp assumed a native-like structure, and hence their defective calreticulin binding is not a consequence of their gross misfolding. For most of the Erp57 b' mutants, no significant binding above the level of the background signal was detected, independent of their capacity to form cross-linking products with calreticulin. This distinction may reflect the more rigorous nature of the pull-down assay.

A comparison of the effects of mutations on substrate binding by PDI (cf. Ref. 18) with the effects of mutations on Erp57-calreticulin interactions is shown in Fig. 7 (B and C). Significantly, all residues whose mutation disrupts PDI-substrate binding also have an effect on Erp57-calreticulin interactions. In both cases, the greatest perturbation is observed after mutation of the equivalent residue within the b' domain, namely Ile<sup>289</sup> in PDI and Phe<sup>299</sup> in Erp57. This strongly suggests that the generalized substrate binding site located in the b' domain of PDI has become specialized in Erp57 for a specific interaction with calreticulin and calnexin. However, although the substrate binding site in PDI appears to be primarily defined by four hydrophobic residues (18), the calreticulin interaction site of Erp57 seems to encompass a larger region on the face of the b' domain (compare magenta and green residues in Fig. 7, B and C). Although different assays are used to monitor the respective interactions, this most likely reflects authentic differences in the association of different PDI family members with their binding partners.

The binding sites of PDI and PDip are both relatively small (18, 27), and the binding of both proteins to model peptides is inhibited by low molecular weight compounds such as 2-propyl-

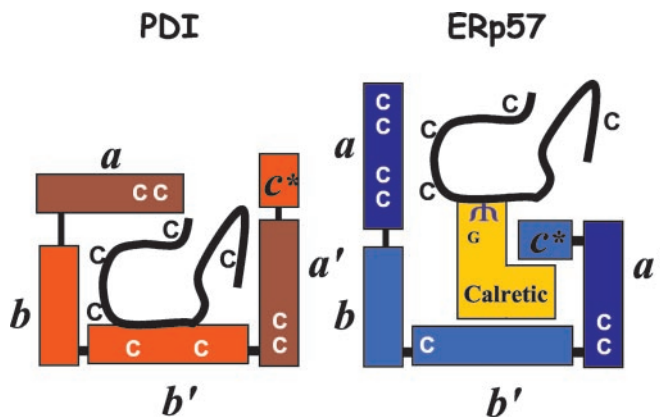


FIG. 9. A comparison of substrate binding to PDI and Erp57. Models indicating the regions of PDI and Erp57 that interact with large substrates and calreticulin respectively are shown. High resolution structures of domains of calnexin (10) and calreticulin (8) suggest that Erp57 binds to the conserved P-loop of these two lectins, and NMR studies confirm this model (11). We propose that Erp57 shows an indirect interaction with its substrate via its interaction with calreticulin, where the b' domain of Erp57 is an essential element of the calreticulin binding site. Our data indicate that the C-terminal extension of Erp57 enhances calreticulin binding significantly, and this is reflected in our working model. In comparison, PDI shows a direct interaction with its substrate, principally through its b' domain, although the a and a' domains contribute to the binding of larger substrates (16, 17).

phenol and 4-propylphenol. In contrast, the binding of Erp57 to the P domain of calreticulin causes a chemical shift or significant decrease in cross-peak intensity in the NMR spectra



for 21 of the residues between Ile<sup>225</sup> and Asn<sup>251</sup> at the tip of the P domain (11), indicating a more substantial binding site than that of PDI or PDip. The tip of the P domain is highly conserved between the human, mouse, *Caenorhabditis elegans*, and *Drosophila* proteins (Fig. 8A), suggesting a similar degree of conservation may be apparent in its binding partner. Inspection of the b' domains of ERp57 from the same species reveals that 31% of the residues are conserved (Fig. 8B). Of particular note are residues Tyr<sup>264</sup>-Ala<sup>287</sup> (71% identity in 24 amino acids), which overlap the region primarily involved in substrate binding to ERp57 (this study) and its equivalent in PDI (18). Significantly, all of the residues in the b' domain where mutation perturbs calreticulin binding lie within this region and are conserved, while the single residue within this region where mutation did not affect the interaction (Glu<sup>270</sup> → Ala) is not conserved. Taken together, these data support a model where the tip of the calreticulin P domain binds to a conserved feature located within the b' domain of ERp57.

Although our study has focused upon the soluble ER lectin, calreticulin, structural studies (8, 9, 11, 28) suggest that in practice our model is likely to depict the molecular basis for the interaction of both calreticulin and calnexin with ERp57. Taken together, our results indicate that ERp57 can be viewed as a specialized version of PDI that binds specifically to two related ER lectins, calnexin and calreticulin, in place of the broad range of substrates that can associate with PDI (*cf.* Fig. 9). These ER lectins would then act to specifically recruit appropriately glucose-trimmed glycoprotein substrates (see Ref. 1) to bind to the complex where they could be acted upon by ERp57 (*cf.* Fig. 9).

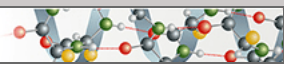
**Acknowledgments**—Our thanks to Kari Kivirikko for encouragement, to Neil Bulleid for the generous gift of recombinant PDI, and to Ben Abell for generating the His-tagged calreticulin construct. We also thank Neil Bullied, Sam Crawshaw, and Martin Pool for helpful comments during the preparation of the manuscript.

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**Protein Structure and Folding:**  
**The Primary Substrate Binding Site in the**  
**b' Domain of ERp57 Is Adapted for**  
**Endoplasmic Reticulum Lectin Association**

PROTEIN STRUCTURE  
AND FOLDING



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*J. Biol. Chem.* 2004, 279:18861-18869.

doi: 10.1074/jbc.M400575200 originally published online February 10, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M400575200](https://doi.org/10.1074/jbc.M400575200)

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